Comparison of the Effects of Moist and Dry Conditions on the Process of Angiogenesis During Dermal Repair

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The effect of moist and dry conditions on the process of angiogenesis during dermal repair was investigated. The moist conditions were achieved by covering excised wounds on porcine flank skin with the adhesive polyurethane dressing Opsite and dry conditions were achieved by exposure to air through dry gauze dressings. Angiogenesis was assessed during the period from 3 to 60 d after injury. Quantitative studies, using computerized image analysis, were carried out on microfocal x-ray images of skin sections whose blood system had been perfused in vivo with a radio-opaque medium. The analytical technique yielded information with regard to vessel number per wound and also the area occupied by blood vessels per unit wound area. Three regions were assessed in each wound bed: upper zone, just below the surface of the wound; the lower zone, just above the base of the wound bed; and the middle zone, midway between the other two zones. The results showed that the wounds maintained in a moist environment revascularized at a greater rate than those maintained in a dry environment. This was apparent in all the zones of the wound bed examined. The development of new vessels occurred in a more orderly manner in the moist wounds. There was an early increase in vessel number rising to a peak around days 3–5, then a gradual decrease in number starting around day 7. In contrast, in the dry wounds the development of blood vessels was less rapid. Peak vessel number in the upper zone was significantly less than that achieved in the moist wounds, and was not reached until 7 d after injury. The decrease in vessel number from the peak was less rapid in the dry wounds, suggesting that there was a delayed entry into the remodeling phase in comparison with the moist wounds. The results also showed that the total percentage area of the wound bed occupied by blood vessels was greater in the moist wounds than the dry wounds from 3 d after injury until day 7. This level of vascularization was maintained beyond 7 d after injury even when the vessel number in the moist wounds was significantly less than in the dry wounds, suggesting that the vessels in the moist wounds were larger and, presumably, more mature. In general, moist wounds showed a more rapid decline towards uninjured skin levels of vascularization than dry wounds. *J Invest Dermatol 99:729–733, 1992*

It has been demonstrated both experimentally and clinically that wounds re-epithelialize more rapidly under moist conditions than under dry [1–5]. Moist conditions also increase the rate of dermal repair [6]. Angiogenesis, the development of new blood vessels, is a vital part of wound healing, re-establishing circulation at the injury site, thereby limiting ischemic necrosis and permitting repair [7].

There are a number of factors that influence the rate of blood vessel growth, including the nature of the wound [8–11], mechanical factors [12–14], intercellular interactions [15,16], the extracellular matrix [17–19], and the presence of growth factors [20–22]. One of the major factors influencing the rate of angiogenesis is the environment maintained over the surface of the wound bed [23,24]. Traditional methods of wound management have involved keeping wounds dry using gauze dressings. However, the importance of maintaining a moist environment over the wound is now being realized and appropriate dressings that achieve this have been developed [25].

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The purpose of this study was to find out if the maintenance of a moist environment over a wound could influence the rate of angiogenesis. A moist environment was provided by covering excised, full-thickness porcine wounds with a semi-occlusive dressing, Opsite, and a dry environment by covering other, similar, wounds with dry gauze, the latter allowing the wound surface to become desiccated.

Angiogenesis was assessed by means of analysis of high-resolution microradiographs of 200-μm–thick sections of the wound bed and adjacent intact skin following perfusion of the blood vessels with a radio-opaque medium [26], a procedure termed microangiography.

MATERIALS AND METHODS

Wounding The wounds investigated were 25 × 25 mm² full-thickness excised lesions made in the flank skin of anesthetized female large white pigs weighing 33–39 kg at the time of wounding. The surgical procedure and application of the wound dressings was that described by Dyson et al [6] as is their pre- and post-operative treatment. Nine pigs were used in the study, one for each time period post-operation, 0 (uninjured skin), 3, 5, 7, 10, 14, 21, 40, and 60 d. Each animal had six wounds made per flank, giving a total of 108 wounds available for assessment of angiogenesis, 54 exposed to

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dry and 54 to moist conditions. Dry conditions were achieved by covering the wounds with dry gauze, raised above the wound bed so that it did not adhere to the wounds, and moist conditions were achieved by covering the wounds with Opsite (Smith and Nephew Medical Ltd.).

Animal Perfusion At the end of each test period the animals were anesthetized with halothane, oxygen, and nitrous oxide via a face mask, intubated, and then maintained on closed circuit anesthesia using a mixture of halothane, oxygen and nitrous oxide. The animals were placed on their backs and a cannula was inserted into the bicarotid trunk extending just past the aortic arch. The internal jugular vein was cannulated as a drain. The animals were then placed in the prone position and 5000 units of heparin were injected into an ear vein. The blood was replaced by infusing 3 l of heated saline (37°C) into the animals via the arterial cannula at a rate of 150–200 ml/min, after which the animals were perfused with 3 l of a radio-opaque medium (60% Baritop 100 [Concept Pharmaceuticals Ltd.], 40% normal saline, and 5% gelatin) at the same rate and temperature. On completion of the perfusion the cannulae were tied off. The animals died while anesthetized. Packs of ice were then placed over the wound sites to aid setting of the gelatin and thereby keep the radio-opaque medium in the vessels.

Tissue Processing After the gelatin had set, the wounds were excised together with about 1 cm of the surrounding intact tissue and fixed in 10% formal saline. After fixation the tissue was dehydrated through a series of alcohols and embedded in paraffin wax. Two-hundred-micrometer vertical sections were cut from each block of tissue and examined by microfocal radiography as described below.

Microfocal Radiographic Technique All the radiographs were produced by a Philips PW 1720 X-ray generator. An aluminum step wedge was x-rayed along with each specimen to allow standardization during the analytical procedure. The film used was Kodak Pelicula SO-343. The exposure details used in this study were as follows: accelerating potential, 30 kV; filament current, 40 mA; target composition, copper; and exposure time, 4 min.

After the films were exposed they were placed in developer (Agfa G153) for 1 min at 20°C. The films were then rinsed in running tap water, placed in fixative (Agfa G354) for 2 min at 20°C, transferred to running tap water for 30 min and dried, and the resulting image examined.

Computerized Image Analysis Each radiograph was digitized at 1024 by 1024 square pixels. The digitization was performed using a CCD camera (Videk Megaplus model K00792) and a 55-mm Nikon Nikkor lens with the radiograph backlit on a lightbox. The camera data were recorded using an IBM PC-AT compatible computer and the Videk Megaplus system. This is comprised of a Matrox frame grabber board and Megagrab software to capture the image, and an Imagagraph display board and Imagepro 1000 software (Media Cybernetics, MD). The aperture and shutter speed of the camera were varied so that the data just filled the range of the 8-bit analogue-to-digital converter in the frame grabber board. The aperture was kept within the optimum range from f/5.6 to f/8.0. A constant pixel size of 0.03 mm was used. Using PC-NFS software and an ethernet card, images were saved to SUN-3 or SUN-4 file-servers on the medical school ethernet. Programs written in C on a SUN 3/60C workstation were used to calculate the vessel number per wound and also the percentage of the area of the wound bed that was occupied by blood vessels.

The software design used operates as follows. Rectangular regions of the image were delineated and using fast Fourier transforms and the edge-detection theory of Marr and Hildreth [27] the blood vessels were segmented out from the background tissue. From this segmented area, the percentage covered by blood vessels was calculated and, by drawing a line across the region of interest, the number of blood vessels crossing the line was calculated. This edge detection works by a method that mimics the human visual system and subtracts a blurred version of the image away from the original image.

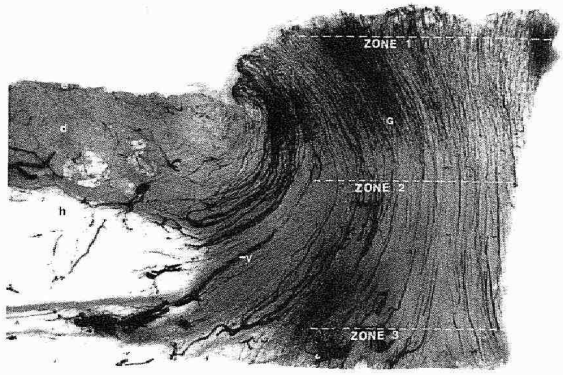

Figure 1. Microangiogram of a wound illustrating the position of the measurement zones. d, dermis of uninjured skin adjacent to the wound bed; e, epidermis of uninjured skin; G, granulation tissue; h, hypodermis; v, new blood vessel in the wound bed.

Regions that are negative are considered to be background and regions that are positive are foreground, with the zero-crossing between positive and negative marking the edge of objects.

The position of the vessel number measurements made across the wound bed are indicated by the *broken lines* in Fig 1. Vessel-area measurements were carried out in six rectangular areas (500 × 500 μm each) across the same field. The three zones of interest were 1) zone 1, adjacent to the superficial surface of the wound bed; 2) zone 2, midway between the upper zone and the lower zone; 3) zone 3, just external to the hypodermis.

Measurements were also made on uninjured tissue to obtain a baseline figure with which to compare the other data. In the uninjured tissue the three zones were located 1) just deep to the dermo-epidermal junction; 2) just external to the hypodermis; and 3) midway between 1 and 2.

All results obtained were tested for statistical significance using the Student two-sample, two-tailed t test.

RESULTS

Vessel Number The results showing average vessel number per zone per wound are expressed graphically in Fig 2–4. The three sets of data correspond to the three measurement zones. It can be seen that uninjured skin contained relatively few vessels detectable by this technique. In contrast, very soon after injury there was an inva-

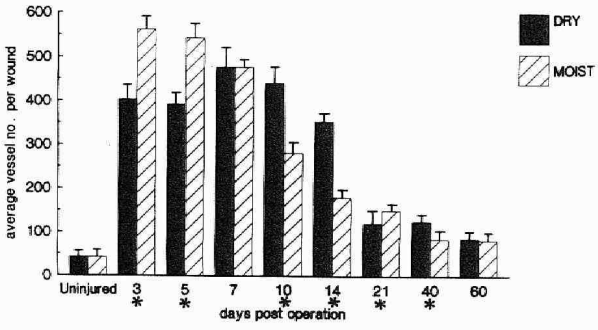

Figure 2. Bar chart showing average vessel number (± standard deviation) per wound against days post operation. Vessel counts carried out in zone 1. * $p < 0.05$.

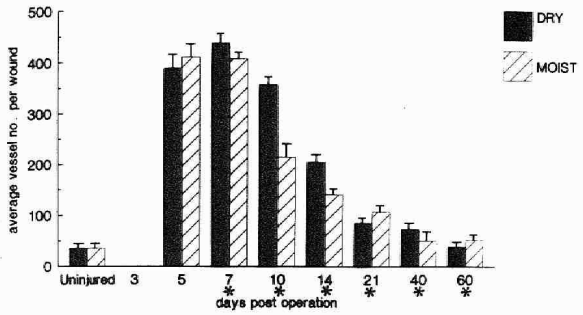

Figure 3. Bar chart showing average vessel number (± standard deviation) per wound against days post operation. Vessel counts carried out in zone 2. * $p < 0.05$.

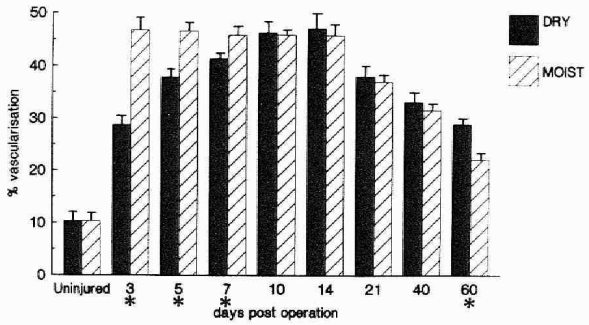

Figure 5. Bar chart showing average vessel area as a percentage of the wound bed area (± standard deviation) against days post operation. Measurements carried out in zone 1. * $p < 0.05$.

sion of new vessels into the wound bed, particularly so in the wounds that were maintained in a moist environment. The vessel number in the upper zone by day 3 post-operation was statistically significantly higher in the moist wounds than in the dry wounds. From day 7 to day 14 vessel number decreased more rapidly in the moist wounds than in the dry wounds in all three zones. This rapid decrease in vessel number in the moist wounds was evident in all three zones analyzed. In zone 1, the vessels in the dry wounds did not begin to decrease in number until day 7. In the moist wounds there was a decrease in vessel numbers from day 3 (zone 1), day 5 (zone 2), and day 7 (zone 3).

Percentage Area of Vascularization The results showing percentage wound area occupied by blood vessels are shown in Figs 5–7. Variation in area with time showed that the area increased more rapidly in the moist wounds than in the dry wounds. The maximum level was reached by day 3 in the moist wounds and remained virtually constant until it showed a significant decrease after day 14.

All points of statistical significance are indicated on the figures with *asterisks*.

DISCUSSION

Wound healing can be conveniently divided into three overlapping phases: inflammation, proliferation, and remodeling or maturation [28]. Each phase is characterized by the presence at the wound site of specific, temporarily resident, cell types. For example, polymorphonuclear leucocytes and macrophages are among the cells that characterize the inflammatory phase, whereas fibroblasts and endothelial cells are predominant during the proliferative phase. The total cellularity and vascularity of the tissue that develops at the wound site increases during the inflammatory and proliferative phases of repair, then decreases during the remodeling phase resulting in scar tissue that is less cellular and less vascular than either the granulation tissue of the proliferative phase or the uninjured dermis. These changes in cellularity and vascularity can be made use of as a means of quantifying the progress of repair. By monitoring, in a differential fashion, the cellularity and vascularity of representative volumes of the wound site at specific times post-injury, for example, by means of cell counts [6] or, as in the present investigation, vessel counts, the rate of repair can be assessed. By comparing the time at which peaks of cellularity and vascularity occur from graphs produced of the data obtained, the efficacy of different treatments can be evaluated. The observation of changes in the time of occurrence of peaks of cellularity [6] formed the basis of our model, which we have now extended to include changes in vascularity. Using the technique of microangiography, high-resolution micrographs were obtained showing the vascular supply to the wound area. Microangiography was developed at the turn of the century as a means of assessing vascularity. The first high-quality microradiographs were produced by Bohatyrschuk in the 1940s [26], using a method that involved the perfusion of the vessels with a radio-oqaque contrast medium and the subsequent production of contact microradiographs of the perfused tissue. Using this method Bohatyrschuck obtained magnifications of up to ×100, allowing the visualization of the microcirculation in a range of organs and in tumors. In 1947 Barclay and colleagues, using improved techniques, produced high-resolution

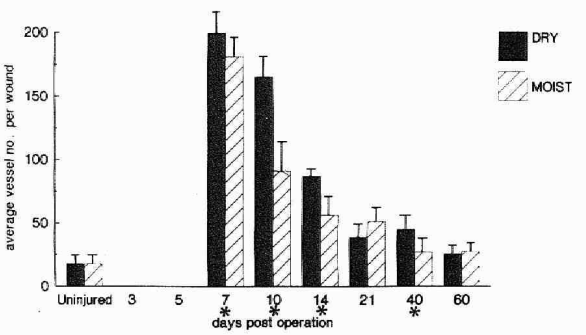

Figure 4. Bar chart showing average vessel number (± standard deviation) per wound against days post operation. Vessel counts carried out in zone 3. * $p < 0.05$.

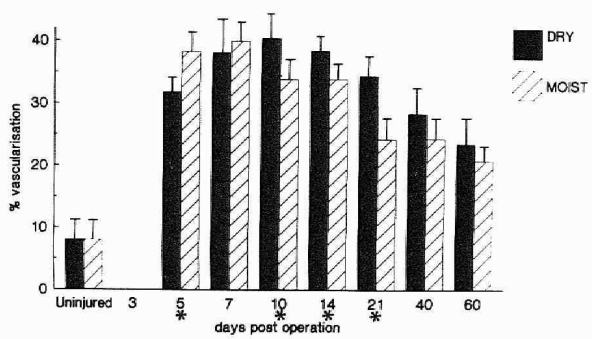

Figure 6. Bar chart showing average vessel area as a percentage of the wound bed area (± standard deviation) against days post operation. Measurements carried out in zone 2. * $p < 0.05$.

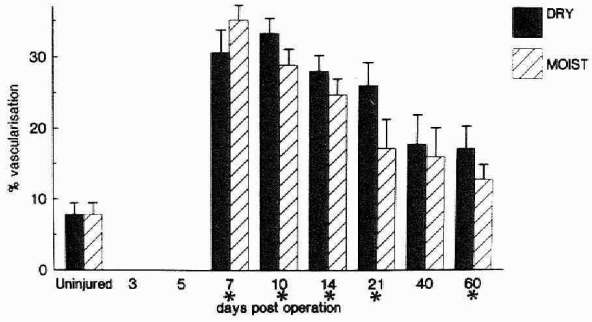

Figure 7. Bar chart showing average vessel area as a percentage of the wound area (± standard deviation) against days post operation. Measurements carried out in zone 3. * $p < 0.05$.

microradiographs of the blood vessels of many tissues and organs of the body. Since then the technique has been used to demonstrate the vessels of organs such as the stomach [29], the testis [30], and the skin, both intact [31,32] and during healing [33]. It permits the visualization of the components of the microcirculation, vessel branching, the anatomical relationship between vessels of different caliber, and the distribution of these vessels in and around the site of injury. Computer-assisted image analysis of the microradiographs allows quantitative information to be obtained. Microangiography has advantages over histology as a means of monitoring angiogenesis; larger volumes of tissue can be sampled and, because only communicating blood vessels are identified after perfusion in vivo, it permits the comprehensive demonstration of the spatial relationship between the functional, patent, vessels of the microcirculation. Traditional morphometric histologic techniques involve assessments of smaller volumes of tissue, do not demonstrate communication between the components of the microcirculation, and, if they involve the identification of endothelial cells and/or of basement membrane components, do not distinguish between blood vessels and lymphatics, nor do they indicate which vessels are patent. It has been shown that endothelial cell-count data obtained from histologic sections can be a misleading index of angiogenesis [6]. The wound bed may appear very well vascularized yet the endothelial cell number can be extremely low; this is probably related to the shape and orientation of the cells, which results in few being sectioned in the samples of tissue examined. The development of suitable image-analysis hardware and software allowed us to gather the data necessary to carry out the comparison of treatment regimes.

The results obtained showed that angiogenesis occurred more rapidly under moist than dry conditions. The vessels showed an earlier entry into the remodeling phase in the moist wounds as evidenced by the rapid decrease in vessel number by 7 d after injury. In the dry wounds the decline in vessel number from day 7 was not as rapid as that seen in the moist wounds. Although vessel number decreased in the moist wounds from day 7 (see Fig 2), the percentage of the wound area occupied by blood vessels remained virtually constant until day 14 when the moist wounds showed a significant decrease in this percentage. It can be deduced from this that the vessels must be of greater diameter than those in the dry wounds. This indicates a greater degree of vascular maturity in the moist wounds, associated with more rapid development of larger arterioles, arteries, venules, and veins from the capillary plexus formed in the initial stages of angiogenesis.

Examination of photographs taken of the wounds throughout the repair period indicate that contraction appeared to be more rapid in the moist wounds than in the dry wounds. If vessel number in the moist wounds had remained unchanged throughout the healing period we would have expected to find an increase in vessel number per unit area as the wound contracted; however, as the results show, there was a decrease in vessel number per unit area even though the total area appeared to be contracting rapidly. These observations add further evidence, albeit qualitative, to the quantitative data that suggests that the moist wounds lose blood vessels more rapidly than dry wounds, indicating that they enter the remodeling phase earlier.

Because capillary growth begins during inflammation and continues during proliferation, it has been suggested that it may be initiated by substances released from the cells that are within the wound bed during the inflammatory period, e.g., granular leukocytes [34], lymphocytes [35], and macrophages [36]. It is probable that in the gauze-dressed, dry wounds, a high percentage of the factors released by any or all of these cell types would be removed by absorption into the gauze. However, under a film dressing these soluble factors could collect in the fluid bathing the wound surface and, provided that they remained active, be available to attract endothelial cells towards the superficial surface of the wound. The acceleration of angiogenesis seen in the moist wounds may have been due, in part, to accumulation of factors stimulating angiogenesis such as heparin [37], tumor necrosis factor alpha, etc. [38] under the dressing.

Other conditions that may have contributed to the difference in rates of angiogenesis include 1) the presence of a scab in the dry wounds acting as a physical barrier delaying the entry of vessels, and 2) the adhesive material on the dressing. The adhesive could have been stimulatory, although no reference to this possibility has been found in the literature.

The results obtained in the course of this investigation suggest that the use of a semi-occlusive film dressing to provide moist conditions may enhance the rate of repair by allowing the rapid invasion of the injured area by new blood vessels. It is also suggested that under these conditions the rate of maturation of the vessels is accelerated.

These observations differ from those made by Ksander et al in 1990 [39] on rodent skin wounds, where dry wounds produced more granulation tissue than moist wounds.

Species differences could be involved in the differences in response observed. Wounds in the freely mobile skin used by Ksander et al contract more and granulate less during closure than wounds in the less mobile porcine skin investigated here. In this respect porcine skin is more similar to human skin. By reducing scab formation, which probably mechanically obstructs contraction, the use of occlusive dressings on rodent wounds may permit more contraction, and thus by reducing the dimensions of the wound reduce the amount of granulation tissue required. We suggest that the differences in the contractile ability of rodent and porcine wounds may, at least in part, be responsible for the apparent differences in "healing" observed by us and by Ksander et al. Meyer, Schwarz, and Neurand [40] have considered the suitability of various animal species as a model for human skin. They have shown that the morphologic differences between the skin of mammals with dense hair coats and that of humans make any comparison limited. They conclude that among the domestic species the pig provides the most suitable experimental model for dermatologic research. This, together with the differences in the mechanisms of wound closure referred to above, indicates the greater relevance of wound-healing studies on porcine skin to wound healing in humans and supports our use of this model.

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